RESEARCH ARTICLE

Antibiotic Susceptibility and Differential expression of virulence genes in *Staphylococcus aureus*

Mohammad A. Al-kafaween[1], **Mohammad Abu-Sini**[1], **Hamid A. Nagi Al-Jamal**[2*]
[1] Faculty of Pharmacy, Department of Pharmacy, Al-Zaytoonah University of Jordan, Amman, Jordan
[2] School of Biomedicine, Faculty of Health Sciences, Universiti Sultan Zainal Abidin, Terengganu, Malaysia

Abstract: *Staphylococcus aureus* is one of the most common pathogens in biofilm-associated chronic infections. Bacteria within biofilm are typically more resistant to antibiotics than are planktonic cells. The objective of the study was to evaluate the effect of Ciprofloxacin (CP), Gentamicin (GEN), Tetracycline (TET), Amikacin (AMK), Clindamycin (CLI), Erythromycin (Ery) and Vancomycin (VAN) against *S. aureus*. The effects of CP, GEN, TET, AMK, CLI, Ery and VAN on *S. aureus* planktonic and biofilm were determined by antibiotic susceptibility test, Minimum Inhibitory Concentration (MIC), Minimum Bactericidal Concentration (MBC), microtiter plate and RT-qPCR. Antibiotic susceptibility test showed that CP, GEN, TET, AMK, CLI, Ery and VAN have antibacterial activity against *S. aureus* with an inhibition zone of 28 mm, 21 mm, 27 mm, 20 mm, 25 mm, 27 mm and 19 mm respectively. The results showed that the CP and AMK possessed the lowest MIC and MBC values with 0.125 μg/mL and 0.25 μg/mL respectively against *S. aureus*. All the tested antibiotics at MIC concentration were found to disrupt microcolony formation in *S. aureus*. In addition, at 0.25 μg/mL concentration to 8 μg/mL concentration of each antibiotic were significantly found to degrade and inhibit biofilm formation in *S. aureus*. The RT-qPCR analysis showed that four genes *argF*, *purC*, *adh*, and *fabG* in *S. aureus* were downregulated, whilst, three genes *scdA*, *pykA* and *menB* were upregulated after exposure to CP, GEN, TET, AMK, CLI, Ery and VAN. This study showed the activity of all antibiotics against planktonic, biofilm and gene expression profile in *S. aureus* and that different concentrations of antibiotics have different degrees of potential effect on planktonic, biofilm and gene expression. These results provide the theoretical parameters for the selection of effective antimicrobial in clinical therapy and demonstrate how to correctly use antibiotics at MIC and sub-MIC as preventive drugs.
Keywords: *Staphylococcus aureus*, antibiotics, biofilm, differential expression, RT-qPCR, virulence genes

Correspondence to: Hamid A. Nagi Al-Jamal, School of Biomedicine, Faculty of Health Sciences, Universiti Sultan Zainal Abidin, Terengganu, Malaysia; E-mail: aljamalhamid@unisza.edu.my

Received: March 28, 2022; **Accepted:** May 3, 2022; **Published Online:** May 10, 2022
Citation: Al-kafaween, M.A., Abu-Sini, M. and Al-Jamal, H.A.N., 2022. Antibiotic Susceptibility and Differential expression of virulence genes in *Staphylococcus aureus*. *Applied Environmental Biotechnology*, 7(1): 6-15. http://doi.org/10.26789/AEB.2022.01.002
Copyright: Antibiotic Susceptibility and Differential expression of virulence genes in *Staphylococcus aureus* © 2022 Mohammad A. Al-kafaween, Mohammad Abu Sini and Hamid A. Nagi Al-Jamal. This is an Open Access article published by Urban Development Scientific Publishing Company. It is distributed under the terms of the Creative Commons Attribution-Noncommercial 4.0 International License, permitting all non-commercial use, distribution, and reproduction in any medium, provided the original work is properly cited and acknowledged.

1 Background

A biofilm is an assemblage of one or more types of microorganisms attached to a surface, unlike in the case of planktonic in which bacteria exist as individual organisms (Al-kafaween and Al-Jamal, 2022; Tarawneh et al., 2021). Biofilm formation not only avoids human immune surveillance but is also known to reduce antibiotic penetration. There is a positive relationship between drug resistance and biofilm development in *Staphylococcus aureus* isolates (Tahaei et al., 2021). Biofilm-forming bacteria are known to exhibit approximately 100-1000 times higher antibiotic resistance than planktonic bacteria (Huwaitat et al., 2021; Tarawneh et al., 2021; Al-kafaween and Hilmi, 2022).

The reasons for this are as follows: (A) the penetration of antibiotics into the biofilm is poor; (B) bacteria in the biofilm state are relatively slower in growth and proliferation than those in the planktonic state; (C) biofilms are different from planktonic bacteria in specific metabolic processes; and (D) biofilms appear to facilitate horizontal gene transfer of antibiotic resistance genes (Khan et al., 2020). Infections caused by biofilm-forming pathogens are difficult to treat using conventional methods and have become a serious problem worldwide (Al-Kafaween et al., 2020; Khan et al., 2020). For developing therapeutic agents for biofilm-forming bacterial infections, the susceptibility of antibiotics should be tested under biofilm conditions rather than in the planktonic state (Al-Kafaween et al., 2020).

Bacterial strains with varying degrees of biofilm formation are believed to contribute significantly to the search for novel antimicrobial agents effective against chronic biofilm-associated infections and to understanding the mechanisms of antibiotic resistance due to biofilm development. *Staphylococcus aureus* is a major cause of community-acquired and hospital infections, can form biofilms on the surfaces of various medical devices used in hospitals, resulting in chronic

and persistent infections (Al-kafaween et al., 2021; Shrestha et al., 2019). Treatment of infection associated with *S. aureus* is severely limited due to reduced sensitivity to antibiotics. One of the most important problems with the formation of biofilms by *S. aureus* is the development of antibiotic resistance (Kranjec et al., 2021; Shin et al., 2021; Al-kafaween et al., 2021). Although the mechanism of *S. aureus* biofilm formation remains unclear, it is believed that bacteria deposit on solid surfaces, accumulate multi-layered cell clusters, and then evolve into mature biofilms (Moormeier and Bayles, 2017). Therefore, the purpose of the study was to investigate the effects of Ciprofloxacin (CP), Gentamicin (GEN), Tetracycline (TET), Amikacin (AMK), Clindamycin (CLI), Erythromycin (Ery) and Vancomycin (VAN) against *S. aureus*.

2 Materials and Methods

2.1 Bacterial Strains and Growth Conditions

A reference strain of *Staphylococcus aureus*(ATCC-6538) was purchased from the American Type Culture Collection (ATCC).*Staphylococcus aureus* (ATCC 6538) was grown on nutrient agar (NA) plates at 37°C for 24 hours. Then, 2-5 colonies from agar plate were picked and cultured in Mueller Hinton Broth (MHB) or Tryptic Soy Broth (TSB) for 24 hours at 37°C. After incubation, 1.2 ml of culture was added to the 2 ml vial (30% glycerol final) and the mixture were inverted for 10 seconds. Then, the samples were stored in the freezer at -80°C (Djahmi et al., 2013; Silva et al., 2021).

2.2 Antibiotic susceptibility testing

S. aureus was assessed for antimicrobial susceptibility by the disk diffusion method according to the Clinical and Laboratory Standards Institute (CLSI) guidelines. A few single colonies of *S. aureus* were aseptically picked from the fresh culture from NA agar plate using sterile cotton swab and then were suspended into 10 mL of saline solution. The inoculum concentration was adjusted to 0.5 McFarland. A sterile cotton swab was then dipped into the bacterial suspension and were rotated onto the tube with firm pressure to remove excess fluid. The swab was streaked over the entire surface of MHA agar plate for three times and each time the plate was rotated approximately 90° to ensure even distribution. The antibiotics tested were ciprofloxacin (5 μg), gentamicin (10 μg), tetracycline (30 μg), amikacin (30 μg), clindamycin (10 μg), erythromycin (15 μg), and vancomycin (30 μg). Distilled water was used as a negative control. The plates were incubated at 37°C for 24 hours. Digital venire calliper was used to measure the zones of inhibition. The assay was carried out in triplicates (Nassar et al., 2019; Yarbrough et al., 2018).

2.3 Determination of the Minimum Inhibitory Concentration (MIC)

The MIC of the antimicrobials was determined by the method involving micro-dilution in culture broth, as indicated by the Clinical and Laboratory Standards Institute. In this process, different concentrations of CP, GEN, TET, AMK, CLI, Ery and VAN were used. Five colonies of *S. aureus* strain were taken from the NA plates, inoculated into 5 mL of TSB, and incubated at 37°C for 24 hours. In this experimental work, microtiter plates were used and *S. aureus* suspension with 0.5 McFarland were prepared as described previously. The wells were filled with a 200 μL of inoculum of *S. aureus* and 200 μL of CP, GEN, TET, AMK, CLI, Ery and VAN at a range of concentrations (8 μg, 4 μg, 2 μg, 1 μg, 0.5 μg, 0.25 μg and 0.125 μg/ml). Wells filled with 200 μl of each antibiotics and used as corresponding negative control, wells filled with 200 μL of inoculum as bacterial growth control and finally wells were filled with 200 μl of broth as sterility control. Growth was determined by measuring the optical density of each sample at 570 nm wavelength using microtiter plate reader (Tecan Infinite 200 PRO, Austria). The value for MIC was set as the minimum concentration of the antimicrobial substance necessary to prevent bacterial growth after 24 h of incubation at 37°C. The experiment was performed in triplicate (Al-Bakri and Mahmoud, 2019; Rodríguez-Melcón et al., 2021).

2.4 Determination of the Minimum Bactericidal Concentration (MBC)

The dilution in broth method was used to calculate the MBC for the CP, GEN, TET, AMK, CLI, Ery and VAN. A volume of 0.1 mL was removed from the wells in the microtiter plates, where no growth was observed after 24 hours of incubation at 37°C, and was then inoculated onto the surface of NA plates (Oxoid). They were incubated for 24 hours at 37°C, with MBC being taken to be the lowest concentration of the substance at which no colonies formed under these conditions. Plate free of any bacterial growth was recorded as the MBC value. The experiment was carried out in triplicate (Rodríguez-Melcón et al., 2021).

2.5 Microcolony formation

For visualization of microcolony by light microscopy, *S. aureus* suspension with 0.5 McFarland were prepared as described previously. Two hundred microlitre of the culture was transferred into glass coverslips in 24-well plates and incubated for 24 hours at 37°C. After incubation, 200 μL of planktonic cells were removed and replaced with 100 μL MIC of each antibiotic (CP, GEN, TET, AMK, CLI, Ery and VAN) for 24 hours at 37°C. Inoculum without antibiotic was served as positive control. The coverslips were with phosphate-buffered saline (PBS) and were stained with 0.2% (w/v) crystal violet. Images were viewed by light microscopy

using oil immersion at 100x magnification. The procedure was repeated in triplicate (Kaur et al., 2012; Al-kafaween et al., 2021; Rodríguez-Melcón et al., 2021).

2.6 Biofilm inhibition assay

To determine the concentration of antibiotics (CP, GEN, TET, AMK, CLI, Ery and VAN) require to inhibit a biofilm formation of *S. aureus*, the microtiter plate method was used; a range of antibiotics concentrations (8 μg, 4 μg, 2 μg, 1 μg, 0.5 μg, 0.25 μg and 0.125 μg/ml) were freshly prepared from a stock solution. *S. aureus* cultures was adjusted to be 0.5 McFarland standard. Approximately 100 μl of each concentration of antibiotics was dispensed into wells of 96-well plates and inoculated with 200 μl of *S. aureus*. A 200 μl of each antibiotic were served as corresponding negative control, 200 μl of inoculum without added antibiotics were used as positive control and 200μl of broth was served as sterility control. The plates were incubated for 24 hours at 37°C. The media was removed by invertip the plate. The plates were washed with PBS to remove planktonic and drained for drying. The plates were stained with 200 μl of 0.2% crystal violet for 6 min. Then, the plates were rinsed under running tap water, air dried at room temperature before solubilizing the biofilm with 95 of ethanol. The absorbance was measured by using a microplate reader at 570 nm. The percentage of biofilm inhibition was calculated by following formulas as mentioned below. The experiment was repeated in triplicate (Gomes et al., 2011; Kaur et al., 2012; Al-kafaween et al., 2021; Resch et al., 2005; Rodríguez-Melcón et al., 2021; Al-kafaween et al., 2019).

2.7 Biofilm degradation assay

To determine the concentration of antibiotics (CP, GEN, TET, AMK, CLI, Ery and VAN) required to degrade a biofilm of *S. aureus* a microtiter plate method was used as described previously. *S. aureus* cultures was adjusted to be 0.5 McFarland standard. Two hundred microliter of the culture was transferred into wells of 96-well plates and the plates were incubated for 24 hours at 37°C without shaking. Wells containing only bacterial culture was served as positive control, wells containing antibiotics only was used as corresponding negative control and wells with 200 μl of broth was served as sterility control. After 24 hours of incubation, planktonic cells were removed and then 200 μl of different concentrations of antibiotics (8 μg, 4 μg, 2 μg, 1 μg, 0.5 μg, 0.25 μg and 0.125 μg/ml) were added to the wells. Then, the plates were incubated overnight. After incubation time was done, the plates were washed with PBS and the plates were fixed with 200 μl of 2.5% glutaraldehyde for 5 minutes. Then, the plates were washed with PBS. The attached cells or biofilm were then stained with 200 μl of 0.2% crystal violet for 5 minutes and washed again with PBS. Absorbance was determined at 570 nm using microtitre plate reader. This assay was repeated in triplicate. The percentage of biofilm degradation was calculated by following formulas as mentioned below (Gomes et al., 2011; Kaur et al., 2012; Al-kafaween et al., 2021; Resch et al., 2005; Rodríguez-Melcón et al., 2021).

$$\text{Biofilm (\%)} = \frac{\text{OD (positive control)} - \text{OD (treatment)}}{\text{OD (positive control)}} \times 100\%$$

2.8 Gene expression profile (RT-qPCR)

Inoculum of *S. aureus* was adjusted to be 0.5 McFarland standard. Then, two hundred microliter of the culture was dispensed into 96-well plate and incubated for 24 hours at 37°C. After incubation, 100 μL of planktonic cells was removed and replaced with 100 μL of MIC of each antibiotic (CP, GEN, TET, AMK, CLI, Ery and VAN) for overnight

Table 1. Genes specific primers were used for RT-qPCR analysis of *S. aureus*

Gene name	Gene name	Annealing temp (C^o)	Primer sequence(5' $\longrightarrow$ 3')
argF	143	52	Forward: CCAAGCAGAATTCGAAGGA Revers: GGATGCGCACCTAAATCAAT
purC	117	62	Forward: GAAGCGCATTTTCTCAACAA Revers: CCCTTACCTGCCATTGTGTC
adh	124	62	Forward: GTTGCCGTTGGTTTACCTGT Revers: TTCAGCAGCAAATTCAAACG
scdA	132	56	Forward: CGAAAGCAGCGGATATTTTT Revers: GCGAACCTGGTGTATTCGTT
pykA	126	52	Forward: TGCAGCAAGTTTCGTACGTC Revers: GGGATTTCAACACCCATGTC
menB	109	56	Forward: CTGGGGAAGGTGATTTAGCA Revers: ACCGCCACCTACAGCATAAC
fabG	122	54	Forward: CCGGGACAAGCAAACTATGT Revers: CCAAAACGTGCTAACGGAAT
yqiL*	125	62	Forward: GACGTGCCAGCCTATGATTT Revers: ATTCGTGCTGGATTTTGTCC

Note: *yqiL was used as a reference gene.

Table 2. Zones of inhibition dimeter (mm) of the antibiotics against *S. aureus*

Antibiotics	Tested Organism *Staphylococcus aureus*			Main value
	1st	2nd	3rd	
Ciprofloxacin (CP)	28 mm ± 1.2	28 mm ± 1.2	28 mm ± 1.2	28 mm ± 1.2
Gentamicin (GEN)	22 mm ± 0.9	21 mm ± 0.9	20 mm ± 0.9	21 mm ± 0.9
Tetracycline (TET)	28 mm ± 1.1	27 mm ± 1.1	27 mm ± 1.1	27 mm ± 1.1
Amikacin (AMK)	20 mm ± 0.5	21 mm ± 0.5	20 mm ± 0.5	20 mm ± 0.5
Clindamycin (CLI)	25 mm ± 1.0	25 mm ± 1.0	25 mm ± 1.0	25 mm ± 1.0
Erythromycin (Ery)	27 mm ± 1.1	27 mm ± 1.1	26 mm ± 1.1	27 mm ± 1.1
Vancomycin (VAN)	19 mm ± 0.4	19 mm ± 0.4	19 mm ± 0.4	19 mm ± 0.4

Note: Ciprofloxacin (CP), Gentamicin (GEN), Tetracycline (TET), Amikacin (AMK), Clindamycin (CLI), Erythromycin (Ery) and Vancomycin (VAN). Mean ±standard deviation (SD), n = 3.

at 37°C. Inoculum without antibiotic was used as positive control. The mixture was resuspended in 1000 μl PBS and vortexed for 1 minute to break up cell aggregation. This mixture was centrifuged at 13,000 rpm for 1 minute. The supernatant was discarded and the pellet was washed twice in phosphate buffered saline. The total RNA from treated and untreated of *S. aureus* was extracted using the SV Total RNA Isolation System (Promega, UK). RNA concentration (ng/ml) and purity (OD_{260} nm/ OD_{280} nm and OD_{260} nm/ OD_{230} nm) were assessed by spectrophotometric measurements using a NanoDrop device. This examination showed constant RNA purity for all degradation steps that indicating the degradation levels was successful. Only sample ranged between 1.8 and 2.1 being accepted for conversion to cDNA. RNA was stored at -80°C. Samples were adjusted to 100 ng/μl to convert to cDNA. Total RNA samples was converted to cDNA by using a cDNA conversion kit (Promega, UK). The RT-qPCR mastermix for each reaction was prepared based on the manufacturer's instructions (Promega, UK). Sequences of the primers for *S. aureus* were identified from previous studies as shown in Table 1. Lyophilised and desalted oligonucleotides were reconstituted using sterile ultra-pure water. Oligonucleotides of *S. aureus* were optimised and the efficiencies were tested prior to gene expression analysis. Densitometry was performed by using the Applied Biosystems StepOne Software v2.3. To determine the level of relative gene expression of *S. aureus* samples, a modified $2^{-\triangle\triangle}$ Ct method was used. The experiment was performed in triplicate (Al-Kafaween et al., 2020; Al-kafaween et al., 2021; Jarrar et al., 2019; Jarrar et al., 2022; Livak and Schmittgen, 2001; Maddocks et al., 2012; Roberts et al., 2012; Schmittgen and Livak, 2008; Wasfi et al., 2016; Yadav et al., 2012).

2.9 Statistical Analysis

Data was presented as mean ± standard deviation. Independent student t-test from SPSS version 20 was used to compare between treated and control groups. The significant was set at $P<0.05$.

3 Results

3.1 Antibiotic susceptibility testing

Antibiotic susceptibility of *S. aureus* showed varying degrees of susceptibility patterns against the antimicrobial agents. Antibiotic susceptibility test showed that CP, GEN, TET, AMK, CLI, Ery and VAN has antibacterial activity against *S. aureus* with an inhibition zone of 28 mm, 21 mm, 27 mm, 20 mm, 25 mm, 27 mm and 19 mm respectively. The most antibiotics effective on *S. aureus* were CP and Ery.

3.2 Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)

As shown in Table 3 values for MBCs were greater than those for MICs with respect to the strain tested. Moreover, considerable differences were observed between antibiotics against *S. aureus*. The recorded values for MBCs were 0.25 μg/ml for CP and AMK for *S. aureus* strain, 0.5 μg/ml for VAN, TET and GEN for *S. aureus* and 1.0 μg/ml for Ery and CLI for *S. aureus*. Notably, CP and AMK demonstrated considerable efficacy, as shown by the low values for MIC; 0.125 μg/ml and MBC; 0.25 μg/ml for *S. aureus* (Table 3).

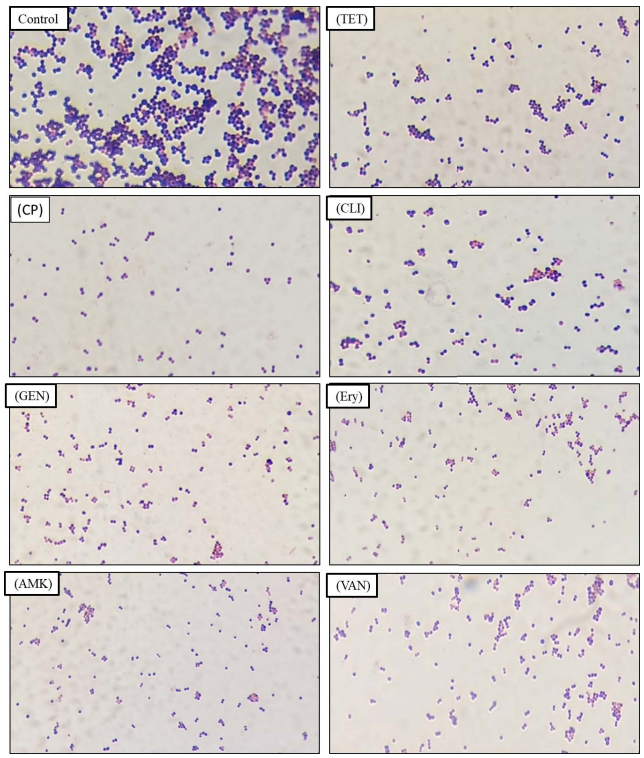
Table 3. Minimum inhibitory concentration (MICs) and minimum bactericidal concentration (MBCs) of seven antibiotics for *S. aureus*

Antibiotics	MIC (μg/mL)	MBC (μg/mL)
CP	0.125	0.25
GEN	0.25	0.5
TET	0.25	0.5
AMK	0.125	0.25
CLI	1	2
Ery	1	2
VAN	0.25	0.5

Note: Ciprofloxacin (CP), Gentamicin (GEN), Tetracycline (TET), Amikacin (AMK), Clindamycin (CLI), Erythromycin (Ery) and Vancomycin (VAN).

3.3 Microcolony formation

Based on Figure 1, CP, GEN, TET, AMK, CLI, Ery and VAN were found to disrupt microcolony formation in *S. aureus* at sublethal dose concentrations of each antibiotic. This demonstrates the significant impact of CP, GEN, TET, AMK, CLI, Ery and VAN treatment on formation of the early biofilm stage in *S. aureus*. Light microscopy verified that microcolonies formation was disrupted by antibiotics easily at MIC-sublethal dose concentrations (Figure 1).

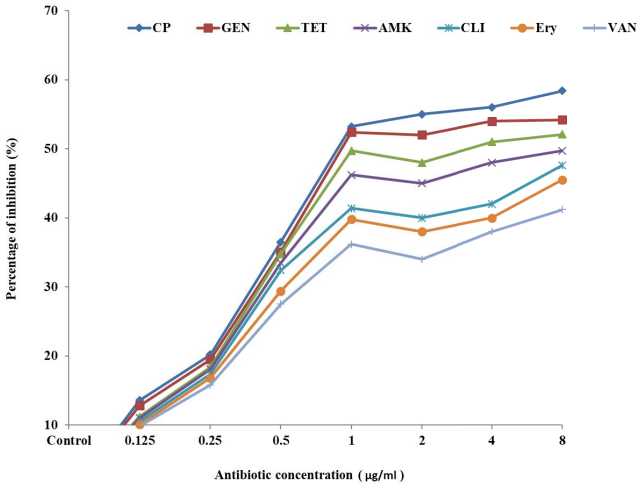


Note: *S. aureus* (Control) and *S. aureus* exposure to Ciprofloxacin (CP), Gentamicin (GEN), Tetracycline (TET), Amikacin (AMK), Clindamycin (CLI), Erythromycin (Ery) and Vancomycin (VAN).

Figure 1. Disruption microcolony of *S. aureus* after treated with antibiotics.

3.4 Biofilm inhibition assay

Growth of *S. aureus* biofilm in the presence of 8 μg, 4 μg and 2 μg, 1 μg, 0.5 μg and 0.25 μg/ml concentrations of each antibiotic (CP, GEN, TET, AMK, CLI, Ery and VAN) was significantly ($P<0.05$) reduced relatively compared with control. Similarly, at 1 μg/ml, 0.5 μg/ml and 0.25 μg/ml concentration, growth of *S. aureus* biofilm was significantly ($P<0.05$) reduced after being treated with each antibiotic. However, the growth of *S. aureus* biofilm was not significantly reduced after added 0.125 μg/ml concentration of each antibiotic. In both instances, biofilm inhibition was decreased after exposure to antibiotics compared to the control. The lowest concentration of each antibiotic that inhibited *S. aureus* forming a biofilm was found to be 0.25 μg/ml. However, the greatest inhibition of *S. aureus* biofilm was found to be 58.4%, 54.2%, 52.1%, 49.7%, 47.6%, 45.0% and 41.2% after exposure to 8 μg/ml of CP, GEN, TET, AMK, CLI, Ery and VAN respectively. Three of the most efficient antibiotics that induced a greater reduction of bacterial biofilm population and demonstrated a broader spectrum range, being active against all strains tested, were CP, GEN and TET (Figure 2).



Note: Ciprofloxacin (CP), Gentamicin (GEN), Tetracycline (TET), Amikacin (AMK), Clindamycin (CLI), Erythromycin (Ery) and Vancomycin (VAN).

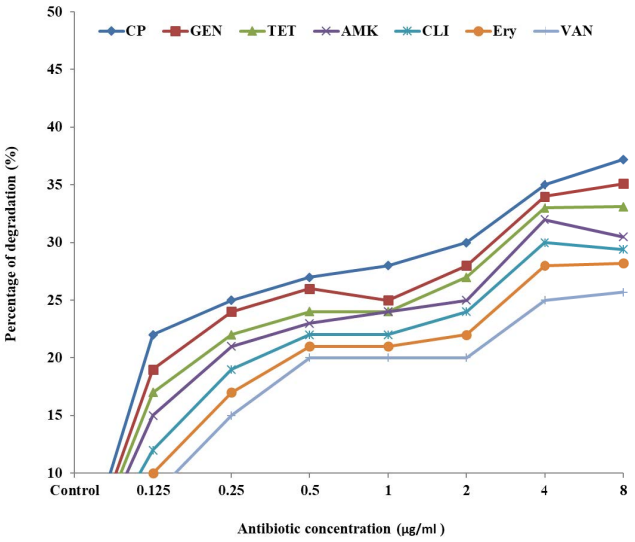
Figure 2. Effect of antibiotics on biofilm formation of *S. aureus*.

3.5 Biofilm degradation assay

The average of optical density (OD) for control sample, tested sample and the degradation of biofilm mass was calculated. Antibiotic was able to degrade the biofilm formation of *S. aureus* compered to control. After 24-hour exposure to several concentration of antibiotics (8 μg, 4 μg, 2 μg, 1 μg, 0.5 μg, 0.25 μg and 0.125 μg/ml), the biofilms were able to reduce 37.2%, 35.1%, 33.1%, 30.1%, 29.4%, 28.2% and 25.7% mass of *S. aureus* after exposure to 8 μg/ml concentration of CP, GEN, TET, AMK, CLI, Ery and VAN respectively. Similarly, each antibiotic was able to reduce 35%, 34%, 33%, 32%, 30%, 28%, and 25% biofilm mass of *S. aureus* at 8 μg/ml concentration of CP, GEN, TET, AMK, CLI, Ery and VAN respectively. The lowest concentration of 0.125 μg/ml of concentration of CP, GEN, TET, AMK, CLI, Ery and VA was found to degrade *S. aureus* biofilm with 22%, 19%, 17%, 15%, 12%, 10% and 8% respectively (Figure 3).

3.6 Genes downregulated in *S. aureus* after exposure to antibiotics

As shown in Figure 4, the expression of four corresponding genes of *S. aureus* did show the level of different expression. Four genes (*argF*, *purC*, *adh*, and *fabG*) had significantly



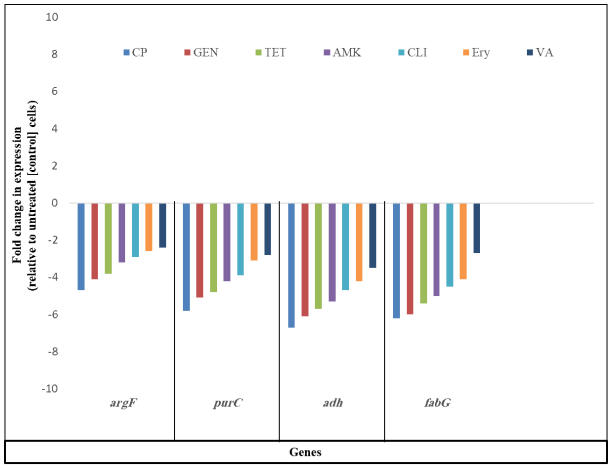
Note: Ciprofloxacin (CP), Gentamicin (GEN), Tetracycline (TET), Amikacin (AMK), Clindamycin (CLI), Erythromycin (Ery) and Vancomycin (VAN).

Figure 3. Effect of antibiotics on biofilm degradation of *S. aureus*.

(P<0.05) decreased levels of expression 4.7-fold, 5.8-fold, 6.7-fold, and 6.2-fold respectively after exposure to CP. Also, *argF*, *purC*, *adh*, and *fabG* had significantly (P<0.05) decreased levels of expression 4.1-fold, 5.1-fold, 6.1-fold, and 6-fold respectively after treated with GEN. In addition, *argF*, *purC*, *adh*, and *fabG* had significantly (P<0.05) decreased levels of expression 3.8-fold, 4.8-fold, 5.7-fold, and 5.4-fold respectively after following treatment with TET. As well as, *argF*, *purC*, *adh*, and *fabG* had significantly (P<0.05) decreased levels of expression 3.2-fold, 4.2-fold, 5.3-fold, and 5-fold after exposure to AMK. The *argF*, *purC*, *adh*, and *fabG* had significantly (P<0.05) decreased levels of expression 2.9-fold, 3.9-fold, 4.7-fold, and 4.5-fold after being treated with CLI. Furthermore, the *argF*, *purC*, *adh*, and *fabG* had significantly (P<0.05) decreased levels of expression 2.6-fold, 3.1-fold, 4.2-fold, and 4.1-fold after treated with Ery. Also, the *argF*, *purC*, *adh*, and *fabG* had significantly (P<0.05) decreased levels of expression 2.4-fold, 2.8-fold, 3.5-fold and 2.7-fold after exposure to VAN (Figure 4).

3.7 Genes upregulated in *S. aureus* after treated with antibiotics

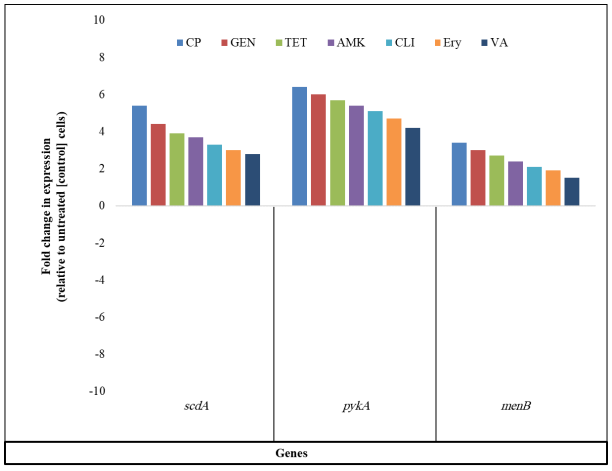
Based in Figure 5, the expression of three genes of *S. aureus* (*scdA*, *pykA* and *menB*) had significantly (P<0.05) increased in a 5.4-fold, 6.4-fold, and 3.4-fold respectively after exposure to CP. Also, *scdA*, *pykA* and *menB* had significantly (P<0.05) increased levels of expression 4.4-fold, 6-fold, and 3-fold respectively after treated with GEN. In addition, *scdA*, *pykA* and *menB* had significantly (P<0.05) increased levels of expression 3.9-fold, 5.7-fold, and 2.7-fold respectively



Note: Ciprofloxacin (CP), Gentamicin (GEN), Tetracycline (TET), Amikacin (AMK), Clindamycin (CLI), Erythromycin (Ery) and Vancomycin (VAN).

Figure 4. Alterations in gene expression profiles in *S. aureus* following treatment with MIC of antibiotics as determined by qPCR.

after following treatment with TET. As well as, *scdA*, *pykA* and *menB* had significantly (P<0.05) increased levels of expression 3.7-fold, 5.4-fold, and 2.4-fold after exposure to AMK. The *scdA*, *pykA* and *menB* had significantly (P<0.05) increased levels of expression 3.3-fold, 5.1-fold, and 2.1-fold after being treated with CLI. Furthermore, the *scdA*, *pykA* and *menB* had significantly (P<0.05) increased levels of expression 3-fold, 4.7-fold, and 1.9-fold after treated with Ery. Also, the *scdA*, *pykA* and *menB* had significantly (P<0.05) increased levels of expression 2.8-fold, 4.2-fold and 1.5-fold after exposure to VAN (Figure 5).



Note: Ciprofloxacin (CP), Gentamicin (GEN), Tetracycline (TET), Amikacin (AMK), Clindamycin (CLI), Erythromycin (Ery) and Vancomycin (VAN).

Figure 5. Alterations in gene expression profiles in *S. aureus* following treatment with MIC of antibiotics as determined by qPCR. Results are expressed as the mean fold change.

4 Discussion

The MIC values for CP, GEN, TET, AMK, CLI, Ery and VAN for *S. aureus* strain of were reported to be below the cut-off point established by the CLSI. However, in recent years a considerable increase has been observed in the prevalence of resistance in bacteria of this microbial species (Argudín et al., 2014; Shin et al., 2021; Van Eldere et al., 2008). Next, we measured the detachment effect of antibiotics on the biofilm of *S. aureus*. Biofilms grown with the high biofilm-forming *S. aureus* strain were incubated with antibiotics. The CP, GEN, TET, AMK, CLI, Ery and VAN showed little biofilm degradation at concentrations below 2 μg/ml. However, all antibiotics at concentrations above 2 μg/ml led to a 20-38% removal of the biofilm. Considering that there is a close correlation between drug resistance and biofilm development, the high biofilm-forming *S. aureus* strain could be very useful for understanding biofilm mechanisms or screening novel antibiotics to eradicate biofilms. Biofilm is a matrix of self-produced extracellular polymeric materials such as exopolysaccharides, proteins, nucleic acids and other substances. Biofilms can play an important role in physical and biological properties such as slow growth or a mechanical barrier and in the development of antimicrobial resistance (Flemming et al., 2016; Fulaz et al., 2019). *S. aureus* is usually used in biofilm related trials as it is adept at forming biofilms (Kwiecinski et al., 2019). And crystal violet staining is the most common assay used to qualify biofilms (Hess et al., 2014; Shin et al., 2021). In this study, a wide range of concentration of antimicrobial agents was selected to detect biofilm formation. In our study, we investigated the in vitro effects of several concentrations of CP, GEN, TET, AMK, CLI, Ery and VAN on biofilm formation in *S. aureus* and we found that all antibiotics were able to inhibit and degrade the biofilm formation of *S. aureus*. Previous studies have been reported that aminoglycoside, fluoroquinolone and polypeptide antibiotics all efficiently prevent *S. aureus* biofilm formation (Qiao et al., 2014; Saising et al., 2012). The results of our study show that *S. aureus* biofilms incubated with CP, GEN, TET, AMK, CLI, Ery and VAN at sub-MIC were significantly inhibited as compared to the control. The results published by Wasfi et al. showed that ciprofloxacin and fluoroquinolone antibiotic, was found to be the most effective in decreasing *S. aureus* and *Enterobacter sp*. biofilm formation (Wasfi et al., 2012). Previous study observed that the antibiotics of ciprofloxacin and amikacin at sub-MIC all reduced *E. coli* biofilm formation in vitro and the inhibitory effects of sub-MIC of agents on biofilm formation was dependent on which antibiotics interfere with the expression of curli fimbriae (Wojnicz and Tichaczek-Goska, 2013). Crystal violet staining was used to investigate the influence of sub-MIC of antibacterial on biofilm formation. Consistent with crystal violet staining and morphological observations, the relative expression levels of biofilm-related genes, as determined by RT-qPCR, followed the same trends. Furthermore, the ratio of biofilm formation on a surface was consistent with changes in expression levels between different drug treatment groups. These outcomes suggested that biofilm formation depends on biofilm-related genes and associated proteins under treatment with sub-MIC of antibiotics. And similar results have been reported previously (Bai et al., 2015; Chen et al., 2016). Nevertheless, the mechanisms by which drugs affect biofilm formation should be investigated further. In this study, the genes *argF*, *purC*, *adh*, *fabG*, *scdA*, *pykA* and *menB* of *S. aureus* were selected to investigate transcription levels using RT-PCR following antibiotic treatment. As demonstrated by RT-qPCR, the expression of *argF*, *purC*, *adh* and *fabG* genes had decreased, while the genes of *scdA*, *pykA* and *menB* were increased in expression of *S. aureus* after exposure to each antibiotic. Such results show the ability of antibiotics in impairing the efficacy of ligand binding that is needed for adherence, demonstrating the improved virulence and biofilm formation in *S. aureus*. Meanwhile, the *agrF* locus comprises a quorum-sensing gene cluster that carries five genes namely *agrB*, *agrD*, *agrC*, *agrA* and *hla*. These genes ease the generation and the discovery of an auto-inducing peptide (AIP) in the regulation of the expression of genes coding for factors of virulence (Alkafaween et al., 2021; Guzman, 2014; Zulkhairi Amin et al., 2018). The decreased expression of *argF*, *purC*, *adh* and *fabG* genes in *S. aureus* following the antibiotics treatment might show that the antibiotics restricts the biofilm formation. Furthermore, our RT-PCR results revealed that the following antibacterial treatment, the expression levels of all genes were in agreement with changes in the biofilm. Also, the levels of *scdA*, *pykA* and *menB* genes in *S. aureus* were increased, while changes in the expression of *scdA*, *pykA* and *menB* will impact the cross-linking of peptidoglycan. *S. aureus* uses multiple layers of genetic regulation to ensure protection from environmental stresses and host defenses; metabolic adaptation to changing nutrient conditions; and coordinated expression of virulence factors. Resistance to active cell wall antibiotics is particularly sensitive to any regulatory changes affecting peptidoglycan metabolism and cell envelope properties. *S. aureus* genomic strain backgrounds can vary by more than 20%, which may contribute to significant strain-specific differences in resistance levels, resistance phenotypes, and resistance gene expression levels.

5 Conclusion

Pathogenic biofilm formation is recognized as a major challenge in treating many persistent infections (Gebreyohannes et al., 2019). Susceptibility testing of planktonic bacteria can be an impediment to the successful treatment of chronic infections caused by biofilm-forming pathogens. In this study, we generated *S. aureus* with different degrees of biofilm formation and measured the MICs and MBCs for low and high biofilm-forming strains. Most importantly, we found a small difference in MICs and MBCs between the antibiotics against *S. aureus*, but the difference in MICs and MBCs

between low and high levels of biofilm formation was significant. We propose that once the biofilm is formed, the bactericidal activity of antibiotics is significantly reduced, regardless of the degree of biofilm formation by *S. aureus*. In this study, we compared patterns of gene expression in *S. aureus* cells treated with and without CP, GEN, TET, AMK, CLI, Ery and VAN. We have also shown that each antibiotic was able to reduce biofilm formation of *S. aureus*. Differential gene expression in response to each antibiotic exhibited downregulation of several genes of *S. aureus*. The obtained results indicate that CP, GEN, TET, AMK, CLI, Ery and VAN may represent promising antibacterial, antibiofilm and anti-virulence agent for treatment and modulation of infections caused by *S. aureus*. A combination of real-time PCR and microarray analysis could be used to verify the findings in this present study. It would be also interesting to look at genes were involved in biofilm formation, quorum sensing and auto-inducers in *S. aureus* and to identify other pathway of gene expression. This could give an idea on level of expression in general. It would certainly be valuable to extend the current study to apply a real-time PCR to detect, identify and quantify the relevant genes of interest between planktonic and biofilm modes of growth to confirm the genes regulation which can be identified by microarray. Next gene sequencing is another the best approach to justify genes regulation.

Funding Sources

This work was supported by Universiti Sultan Zainal Abidin (UniSZA).

Author Contributions

The authors thank all the staff members of the Faculty of Health Sciences and Faculty of Pharmacy at Universiti Sultan Zainal Abidin and Al-Zaytoonah University of Jordan for their support and commitment.

Conflict of Interest

Author declares that there is no conflict of interest in the information presented here.

References

Al-Bakri, A.G., and Mahmoud, N.N., 2019. Photothermal-induced antibacterial activity of gold nanorods loaded into polymeric hydrogel against Pseudomonas aeruginosa biofilm. *Molecules*, 24(14): 2661. https://doi.org/10.3390/molecules24142661

Al-kafaween, M.A., and Al-Jamal, H.A.N., 2022. A comparative study of antibacterial and antivirulence activities of four selected honeys to Manuka honey. *Iranian Journal of Microbiology*, 14(2): 238-251. https://doi.org/10.18502/ijm.v14i2.9193

Al-Kafaween, M.A., Hilmi, A.B.M., Al-Jamal, H.A.N., Elsahoryi, N.A., Jaffar, N. and Zahri, M.K., 2020. Pseudomonas aeruginosa and Streptococcus pyogenes exposed to Malaysian trigona honey in vitro demonstrated downregulation of virulence factor. *Iranian Journal of Biotechnology*, 18(4): e2542.

Al-kafaween, M.A., Mohd Hilmi, A.B., Nagi Al-Jamal, H.A., Jaffar, N., Al-Sayyed, H. and Zahri, M.K. 2021. Effects of Selected Malaysian Kelulut Honey on Biofilm Formation and the Gene Expression Profile of Staphylococcus Aureus, Pseudomonas Aeruginosa and Escherichia Coli. *Jordan Journal of Pharmaceutical Sciences*, 14(1).

Al-kafaween, A.B.M.H., Hamid A.l.I. and Nagi Al-Jamal., 2021. The Beneficial Effects of Stingless Bee Kelulut Honey Against Pseudomonas aeruginosa and Streptococcus pyogenes Planktonic and Biofilm. *Tropical Journal of Natural Product Research*, 5(10): 1788-1796. https://doi.org/10.26538/tjnpr/v5i10.15

Al-kafaween, M.A., Hilmi, A.B.M., Al-Jamal, H.A.N., Al-Groom, R.M., Elsahoryi, N.A. and Al-Sayyed, H., 2021. Potential Antibacterial Activity of Yemeni Sidr Honey Against Pseudomonas aeruginosa and Streptococcus pyogenes. *Anti-Infective Agents*, 19(4): 51-65. https://doi.org/10.2174/2211352519666210319100204

Al-kafaween, M.A., Mohd Hilmi, A.B., Jaffar, N., Al-Jamal, H.A.N., and Zahri, M.K., 2019. Determination of optimum incubation time for formation of Pseudomonas aeruginosa and Streptococcus pyogenes biofilms in microtiter plate. *Bulletin of the National Research Centre*, 43(1): 1-5. https://doi.org/10.1186/s42269-019-0131-9

Al-kafaween, M.A., and Hilmi, A.B.M., 2022. Evaluation of the effect of different growth media and incubation time on the suitability of biofilm formation by Pseudomonas aeruginosa and Streptococcus pyogenes. *Applied Environmental Biotechnology*, 6(2): 19-26. https://doi.org/10.26789/AEB.2021.02.003

Argudín, M.A., Mendoza, M.C., Martín, M.C., and Rodicio, M.R., 2014. Molecular basis of antimicrobial drug resistance in Staphylococcus aureus isolates recovered from young healthy carriers in Spain. *Microbial Pathogenesis*, 74: 8-14. https://doi.org/10.1016/j.micpath.2014.06.005

Bai, J., Wu, Y., Liu, X., Zhong, K., Huang, Y. and Gao, H., 2015. Antibacterial activity of shikimic acid from pine needles of Cedrus deodara against Staphylococcus aureus through damage to cell membrane. *International Journal of Molecular Sciences*, 16(11): 27145-27155. https://doi.org/10.3390/ijms161126015

Capita, R. and Alonso-Calleja, C., 2013. Antibiotic-resistant bacteria: a challenge for the food industry. *Critical Reviews in Food Science and Nutrition*, 53(1): 11-48. https://doi.org/10.1080/10408398.2010.519837

Chen, X., Li, Q., Wang, C., Xu, W., Han, L., Liu, Y. and Wang, J., 2016. Prognostic and diagnostic potential of isocitrate dehydrogenase 1 in esophageal squamous cell carcinoma. *Oncotarget*, 7(52): 86148. https://doi.org/10.18632/oncotarget.13351

Djahmi, N., Messad, N., Nedjai, S., Moussaoui, A., Mazouz, D., Richard, J.-L. and Lavigne, J.-P., 2013. Molecular epidemiology of Staphylococcus aureus strains isolated from inpatients with infected diabetic foot ulcers in an Algerian University Hospital. *Clinical Microbiology and Infection*, 19(9): E398-E404. https://doi.org/10.1111/1469-0691.12199

Flemming, H.-C., Wingender, J., Szewzyk, U., Steinberg, P., Rice, S.A. and Kjelleberg, S., 2016. Biofilms: an emergent form of bacterial life. *Nature Reviews Microbiology*, 14(9): 563-575. https://doi.org/10.1038/nrmicro.2016.94

Fulaz, S., Vitale, S., Quinn, L., and Casey, E., 2019. Nanoparticle-biofilm interactions: the role of the EPS matrix. *Trends in Microbiology*, 27(11): 915-926. https://doi.org/10.1016/j.tim.2019.07.004

Gebreyohannes, G., Nyerere, A., Bii, C., and Sbhatu, D.B., 2019. Challenges of intervention, treatment, and antibiotic resistance of biofilm-forming microorganisms. *Heliyon*, 5(8): e02192. https://doi.org/10.1016/j.heliyon.2019.e02192

Gomes, F., Teixeira, P., Cerca, N., Ceri, H. and Oliveira, R., 2011. Virulence gene expression by Staphylococcus epidermidis biofilm cells exposed to antibiotics. *Microbial Drug Resistance*, 17(2): 191-196. https://doi.org/10.1089/mdr.2010.0149

Guzman, J.D., 2014. Natural cinnamic acids, synthetic derivatives and hybrids with antimicrobial activity. *Molecules*, 19(12): 19292-19349. https://doi.org/10.3390/molecules191219292

Hess, D.J., Henry-Stanley, M.J., and Wells, C.L., 2014. Antibacterial synergy of glycerol monolaurate and aminoglycosides in Staphylococcus aureus biofilms. *Antimicrobial Agents and Chemotherapy*, 58(11): 6970-6973. https://doi.org/10.1128/AAC.03672-14

Huwaitat, R., Coulter, S.M., Porter, S.L., Pentlavalli, S. and Laverty, G., 2021. Antibacterial and antibiofilm efficacy of synthetic polymyxin-mimetic lipopeptides. *Peptide Science*, 113(1): e24188. https://doi.org/10.1002/pep2.24188

Jarrar, Y., Jarrar, Q., Abu-Shalhoob, M. and Sha'ban, E., 2019. Relative expression of mouse Udp-glucuronosyl transferase 2b1 gene in the livers, kidneys, and hearts: the influence of nonsteroidal anti-inflammatory drug treatment. *Current Drug Metabolism*, 20(11): 918-923. https://doi.org/10.2174/1389200220666191115103310

Jarrar, Y.B., Jarrar, Q., Abaalkhail, S.J., Moh'd Kalloush, H., Naser, W., Zihlif, M. and Lee, S.J., 2022. Molecular toxicological alterations in the mouse hearts induced by sub-chronic thiazolidinedione drugs administration. *Fundamental & Clinical Pharmacology*, 36(1): 143-149. https://doi.org/10.1111/fcp.12694

Kaur, S., Harjai, K. and Chhibber, S., 2012. Methicillin-resistant Staphylococcus aureus phage plaque size enhancement using sublethal concentrations of antibiotics. *Applied and Environmental Microbiology*, 78(23): 8227-8233. https://doi.org/10.1128/AEM.02371-12

Khan, F., Pham, D.T.N., and Kim, Y.-M., 2020. Alternative strategies for the application of aminoglycoside antibiotics against the biofilm-forming human pathogenic bacteria. *Applied Microbiology and Biotechnology*, 104(5): 1955-1976. https://doi.org/10.1007/s00253-020-10360-1

Kranjec, C., Morales Angeles, D., Torrissen Mårli, M., Fernndez, L., García, P., Kjos, M. and Diep, D.B., 2021. Staphylococcal biofilms: Challenges and novel therapeutic perspectives. *Antibiotics*, 10(2): 131. https://doi.org/10.3390/antibiotics10020131

Kwiecinski, J.M., Jacobsson, G., Horswill, A.R., Josefsson, E. and Jin, T., 2019. Biofilm formation by Staphylococcus aureus clinical isolates correlates with the infection type. *Infectious Diseases*, 51(6): 446-451. https://doi.org/10.1080/23744235.2019.1593499

Livak, K.J. and Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\triangle\triangle}$CT method. *Methods*, 25(4): 402-408. https://doi.org/10.1006/meth.2001.1262

Maddocks, S.E., Lopez, M.S., Rowlands, R.S., and Cooper, R.A., 2012. Manuka honey inhibits the development of Streptococcus pyogenes biofilms and causes reduced expression of two fibronectin binding proteins. *Microbiology*, 158(3): 781-790. https://doi.org/10.1099/mic.0.053959-0

Manual, O.O.T., 2017. World Organization for Animal Health. Paris, France: 2017. Avian Influenza (infection with avian influenza viruses), 1-23.

Moormeier, D.E., and Bayles, K.W., 2017. Staphylococcus aureus biofilm: a complex developmental organism. *Molecular Microbiology*, 104(3): 365-376. https://doi.org/10.1111/mmi.13634

Nassar, M.S., Hazzah, W.A., and Bakr, W.M., 2019. Evaluation of antibiotic susceptibility test results: how guilty a laboratory could be? *Journal of the Egyptian Public Health Association*, 94(1): 1-5. https://doi.org/10.1186/s42506-018-0006-1

O'Neill, J., 2016. Tackling drug-resistant infections globally: final report and recommendations.

Qiao, Y.-l., Qian, J.-m., Wang, F.-r., Ma, Z.-y. and Wang, Q.-w., 2014. Butyrate protects liver against ischemia reperfusion injury by inhibiting nuclear factor kappa B activation in Kupffer cells. *Journal of Surgical Research*, 187(2): 653-659. https://doi.org/10.1016/j.jss.2013.08.028

Resch, A., Rosenstein, R., Nerz, C. and Götz, F., 2005. Differential gene expression profiling of Staphylococcus aureus cultivated under biofilm and planktonic conditions. *Applied and Environmental Microbiology*, 71(5): 2663-2676. https://doi.org/10.1128/AEM.71.5.2663-2676.2005

Roberts, A.E., Maddocks, S.E. and Cooper, R.A., 2012. Manuka honey is bactericidal against Pseudomonas aeruginosa and results in differential expression. *Microbiology*, 158(12): 3005-3013. https://doi.org/10.1099/mic.0.062794-0

Rodríguez-Melcón, C., Alonso-Calleja, C., García-Fernández, C., Carballo, J. and Capita, R., 2021. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) for twelve antimicrobials (biocides and antibiotics) in eight strains of listeria monocytogenes. *Biology*, 11(1): 46. https://doi.org/10.3390/biology11010046

Saising, J., Dube, L., Ziebandt, A.-K., Voravuthikunchai, S. P., Nega, M. and Götz, F., 2012. Activity of gallidermin on Staphylococcus aureus and Staphylococcus epidermidis biofilms. *Antimicrobial Agents and Chemotherapy*, 56(11): 5804-5810. https://doi.org/10.1128/AAC.01296-12

Schmittgen, T.D. and Livak, K.J., 2008. Analyzing real-time PCR data by the comparative C T method. *Nature Protocols*, 3(6): 1101. https://doi.org/10.1038/nprot.2008.73

Shin, H.-J., Yang, S. and Lim, Y., 2021. Antibiotic susceptibility of Staphylococcus aureus with different degrees of biofilm formation. *Journal of Analytical Science and Technology*, 12(1): 1-7. https://doi.org/10.1186/s40543-021-00294-2

Shrestha, L.B., Baral, R., and Khanal, B., 2019. Comparative study of antimicrobial resistance and biofilm formation among Gram-positive uropathogens isolated from community-acquired urinary tract infections and catheter-associated urinary tract infections. *Infection and Drug Resistance*, 12: 957. https://doi.org/10.2147/IDR.S200988

Silva, V., Almeida, L., Gaio, V., Cerca, N., Manageiro, V., Cania, M. and Poeta, P., 2021. Biofilm formation of multidrug-resistant MRSA strains isolated from different types of human infections. *Pathogens*, 10(8): 970. https://doi.org/10.3390/pathogens10080970

Tahaei, S.A.S., Stjer, A., Barrak, I., Ostorházi, E., Szabó, D. and Gajdács, M., 2021. Correlation between biofilm-formation and the antibiotic resistant phenotype in Staphylococcus aureus isolates: a laboratory-based study in Hungary and a review of the literature. *Infection and Drug Resistance*, 14: 1155. https://doi.org/10.2147/IDR.S303992

Tarawneh, O., Alwahsh, W., Abul-Futouh, H., Al-Samad, L.A., Hamadneh, L., Abu Mahfouz, H., and Fadhil Abed, A., 2021. Determination of Antimicrobial and Antibiofilm Activity of Combined LVX and AMP Impregnated in p (HEMA) Hydrogel. *Applied Sciences*, 11(18): 8345. https://doi.org/10.3390/app11188345

Tarawneh, O., Hamadneh, I., Huwaitat, R., Al-Assi, A.R., and El Madani, A., 2021. Characterization of chlorhexidine-impregnated cellulose-based hydrogel films intended for the treatment of periodontitis. *BioMed Research International*, 20-21 https://doi.org/10.1155/2021/9853977

Van Eldere, J., Vermeersch, P., Van Pelt, K., Verhaegen, J., Kocsis, E., Lagler, H. and Nagy, K., 2008. Comparison of Austrian, Hungarian and Macedonian meticillin-resistant and meticillin-sensitive Staphylococcus aureus strains in relation to accessory gene regulator type.

Wasfi, R., Abd El-Rahman, O., Mansour, L., Hanora, A., Hashem, A. and Ashour, M., 2012. Antimicrobial activities against biofilm formed by Proteus mirabilis isolates from wound and urinary tract infections. *Indian Journal of Medical Microbiology*, 30(1): 76-80. https://doi.org/10.4103/0255-0857.93044

Wasfi, R., Elkhatib, W. F. and Khairalla, A. S., 2016. Effects of selected Egyptian honeys on the cellular ultrastructure and the gene expression profile of Escherichia coli. *PloS One*, 11(3): e0150984. https://doi.org/10.1371/journal.pone.0150984

Wojnicz, D. and Tichaczek-Goska, D., 2013. Effect of sub-minimum inhibitory concentrations of ciprofloxacin, amikacin and colistin on biofilm formation and virulence factors of Escherichia coli planktonic and biofilm forms isolated from human urine. *Brazilian Journal of Microbiology*, 44(1): 259-265. https://doi.org/10.1590/S1517-83822013000100037

Yadav, M.K., Kwon, S.K., Cho, C.G., Park, S.W., Chae, S.W. and Song, J.J., 2012. Gene expression profile of early in vitro biofilms of Streptococcus pneumoniae. *Microbiology and Immunology*, 56(9): 621-629. https://doi.org/10.1111/j.1348-0421.2012.00483.x

Yarbrough, M.L., Lainhart, W. and Burnham, C.-A.D., 2018. Epidemiology, clinical characteristics, and antimicrobial susceptibility profiles of human clinical isolates of Staphylococcus intermedius group. *Journal of Clinical Microbiology*, 56(3): e01788-01717. https://doi.org/10.1128/JCM.01788-17

Zulkhairi Amin, F.A., Sabri, S., Mohammad, S.M., Ismail, M., Chan, K.W., Ismail, N. and Zawawi, N., 2018. Therapeutic properties of stingless bee honey in comparison with european bee honey. *Advances in Pharmacological Sciences*, 18-20. https://doi.org/10.1155/2018/6179596